Express Mail Label No. EL315020614US 09-29-00 A

UTILITY PATENT APPLICATION TRANSMITTAL
(Large Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
BMID9975US

Total Pages in this Submission
64

JC913 U.S. PTO 09/28/00

JC639 U.S. PTO 09/672465 09/28/00

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE

and invented by:

Michael BOTT, Peter DIMROTH and Karin SCHNEIDER

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Enclosed are:

Application Elements

1. ☐ Filing fee as calculated and transmitted as described below

2. ☒ Specification having 27 pages and including the following:

   a. ☒ Descriptive Title of the Invention

   b. ☐ Cross References to Related Applications *(if applicable)*

   c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*

   d. ☐ Reference to Microfiche Appendix *(if applicable)*

   e. ☒ Background of the Invention

   f. ☒ Brief Summary of the Invention

   g. ☒ Brief Description of the Drawings *(if drawings filed)*

   h. ☒ Detailed Description

   i. ☒ Claim(s) as Classified Below

   j. ☒ Abstract of the Disclosure

UTILITY PATENT APPLICATION TRANSMITTAL
(Large Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
BMID9975US
Total Pages in this Submission
64

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   a. ☐ Formal Number of Sheets _____
   b. ☒ Informal Number of Sheets **2**
4. ☒ Oath or Declaration
   a. ☐ Newly executed *(original or copy)* ☒ Unexecuted
   b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☒ With Power of Attorney ☐ Without Power of Attorney
   d. ☐ *DELETION OF INVENTOR(S)*
   Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche *(Appendix)*
7. ☐ Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all must be included)*
   a. ☐ Paper Copy
   b. ☐ Computer Readable Copy *(identical to computer copy)*
   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & document(s))*
9. ☐ 37 CFR 3.73(B) Statement *(when there is an assignee)*
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment `(to follow)`
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
    ☐ First Class ☒ Express Mail *(Specify Label No.):* **EL315020614US**

UTILITY PATENT APPLICATION TRANSMITTAL
(Large Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
BMID9975US
Total Pages in this Submission
64

Accompanying Application Parts (Continued)

15. ☒ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☒ Additional Enclosures *(please identify below):*

General Appointment of Representative for U.S. Patent and Trademark Office Matters.

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims		- 20 =	0	x	$18.00	$0.00
Indep. Claims		- 3 =	0	x	$78.00	$0.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$0.00
OTHER FEE *(specify purpose)* _____						$0.00
					TOTAL FILING FEE	$0.00

☐ A check in the amount of to cover the filing fee is enclosed.

☐ The Commissioner is hereby authorized to charge and credit Deposit Account No. as described below. A duplicate copy of this sheet is enclosed.

- ☐ Charge the amount of as filing fee.
- ☐ Credit any overpayment.
- ☐ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **September 28, 2000**

Signature

Kenneth J. Waite, Reg. No. 45,189
Roche Diagnostics Corporation
9115 Hague Road, Bldg. D
P.O. Box 50457
Indianapolis, IN 46250-0457
Telephone No.: (317) 576-3104
Facsimile No..: (317) 576-2883

cc:

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Michael BOTT, et al.**			Docket No. **BMID9975US**
Serial No. **To Be Assigned**	Filing Date **September 28, 2000**	Examiner **To Be Assigned**	Group Art Unit **To Be Assigned**

Invention: **PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE**

I hereby certify that this **Utility Patent Application**
(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on **September 28, 2000.**
(Date)

Rose Edwards
(Typed or Printed Name of Person Mailing Correspondence)

Rose Edwards
(Signature of Person Mailing Correspondence)

EL315020614US
("Express Mail" Mailing Label Number)

Note: Each paper must have its own certificate of mailing.

Roche Diagnostics GmbH 5234/00/EP

Process for the recombinant production of holo-citrate lyase

The enzyme citrate lyase (EC4.1.3.6) is regarded as a key enzyme of anaerobic citrate degradation and can accordingly be isolated from a number of different prokaryotic cells. The enzyme catalyses the cleavage of citrate into acetate and oxaloacetate. Furthermore it is known that the enzyme complex of the citrate lyase enzyme that has been best examined to date from Klebsiella pneumoniae (formally: Klebsiella aerogenes) is composed of six copies of each of three different subunits and namely an α, β and γ subunit, of a molecular weight of about 550,000 Dalton. In addition it is known that the catalytically active centre is located in the α and β subunit, whereas the γ subunit has the binding site for the prosthetic group 2'-(5"phosphoribosyl)-3'-dephospho CoA. This prosthetic group is bound to the serine residue 14 via a phosphodiester bond.

The citrate lyase enzyme is required in high purity for most applications which are primarily for clinical chemistry and food analysis. Hence the aim is to over-produce the enzyme in an active form in certain host cells by recombinant methods and to isolate it from these cells. Such a process has not yet been described or made known in other ways. Hence citrate lyase is nowadays usually isolated from Klebsiella pneumoniae cells which had been cultured under anaerobic conditions using citrate as the only carbon and energy source. The citrate lyase genes from Klebsiella pneumoniae have been

cloned and sequenced (M. Bott and P. Dimroth, Mol. Microbiol. Vol. 14, 347-356 (1994)). These genes are part of the citC operon which is composed of the five genes citCDEFG. The citC gene codes for citrate lyase ligase which catalyses the formation of an acetyl thioester. The genes citD, citE and citF code for the gamma, beta and alpha subunit of citrate lyase. The protein coded by citG is involved in the biosynthesis of the prosthetic group. Furthermore it is known that the citC operon is induced in the absence of oxygen and in the presence of citrate and Na^+ ions; moreover the expression is strongly dependent on the citA/citB regulation system (M. Bott et al., Mol. Microbiol. Vol. 18, 533-546 (1995); M. Meyer et al., J. Mol. Biol. Vol. 269, 719-731 (1997)).

Expression of the genes coding for citrate lyase from Klebsiella pneumoniae which would preferably be carried out in prokaryotic cells such as E. coli for practical reasons, results in an inactive but nevertheless soluble form of the enzyme (M. Bott and P. Dimroth, Mol. Microbiol. Vol. 14, 347-356 (1994)). The recombinant apo-citrate lyase enzyme can be activated to form the holo-enzyme by subsequent addition of acetyl coenzyme A which is known as a substituent for the acetyl thioester of the native prosthetic group 2'-(5"-phosphoribosyl)-3'-dephospho CoA. However, such an additional activation measure is complicated and laborious. Moreover the necessity to add acetyl CoA is unsuitable for the commercial distribution of citrate lyase or the apo form since the substance decomposes when stored for long periods at 4°C.

Hence the object of the invention is to provide a recombinant, soluble and at the same time active holo-

citrate lyase which eliminates the disadvantages of the known methods.

The object is achieved by a process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organisms whereby the plasmid contains the information of a gene cluster composed of at least six genes and an inducible promoter. The genes comprising the gene cluster code for certain subunits of the protein with citrate lyase activity and/or for a component which participates in the biosynthesis of the complete enzyme. In particular a suitable plasmid contains the genes citC, citD, citE, citF, citG and a DNA fragment that can for example be obtained from E. coli which is located between the genes citF and citG on the E. coli citrate lyase gene cluster. The genes citD, citE and citF code for the corresponding γ, β and α subunits of the enzyme and have molecular weights of about 11,000 Dalton, 32,000 Dalton and 55,000 Dalton. According to the invention it is preferred that one of the genes represents a DNA fragment which codes for a protein containing the motif G(A)-R-L-X-D-L(I)-D-V. A corresponding DNA fragment is particularly preferred which codes for a protein with a molecular weight of about 20,000 Dalton.

In addition it has proven to be advantageous when one gene and optionally a further gene fused to the first gene of the genes comprising the gene cluster is derived from a different organism than the other genes. In particular it has proven to be advantageous when the DNA fragment citX or genes homologous to citX located between citF and citG on the E. coli citrate lyase gene cluster are derived from E. coli, Klebsiella pneumoniae, Haemophilus influenzae or Leuconostoc mesenteroides and

when one or several of the other genes are derived from the microorganism that is specific for the isolated protein having citrate lyase activity which is for example Klebsiella pneumoniae. In Haemophilus influenza, Leuconostoc mesenteroides (S. Bekal et al., J. Bacteriol. Vol. 180, 647-654 (1998)) and Leuconostoc paramesenteroides (M. Martin et al., FEMS Microbiol. Lett. Vol. 174, 231-238 (1999)) the genes citX and citG occur in a fused form. Thus corresponding fusion genes contain the information of two genes. The resulting proteins have a molecular weight of about 52,000 Dalton, have the activities of E. coli CitX and CitG and are thus bifunctional. In the absence of the citX gene or of a gene homologous to citG or of a corresponding citX fusion gene, only the low-molecular apo form (MW 12,000 Dalton, SDS-PAGE) but not the holo form of citrate lyase (MW 14,500 Dalton, SDS-PAGE) could be detected after expression.

According to the invention prokaryotes as well as eukaryotes have proven to be suitable as the host organism. The fact that a soluble active citrate lyase can now be produced in prokaryotes such as e.g. E. coli in a simple manner and in adequate yields without additional activation measures is a major advantage.

Hence it was possible to show that by cloning the entire E. coli citCDEFXG gene cluster under the control of an inducible promoter such as e.g. the lac, lac UV5, T5, tac or T7 promoter, an active enzyme can be expressed having citrate lyase activity even under non-oxygen limiting conditions. Cell extracts containing appropriate expression plasmids result in citrate lyase activities of about 4 to 5 U/mg protein in the cell-free extract whereas cells without recombinant citrate lyase

have no citrate lyase activity when grown aerobically.

In addition the invention concerns the simultaneous expression of the citCDEFG gene cluster from Klebsiella pneumoniae and of the citX gene obtainable from E. coli by which means it is possible to obtain a corresponding citrate lyase in an active form even in prokaryotes and in particular in E. coli.

By this means it was possible to achieve an activity of about 8 U/mg total protein in a cell-free extract under aerobic growth conditions.

The holo-enzyme is purified by methods known to a person skilled in the art. About 100 to 120 µg soluble protein with citrate lyase activity can be obtained from about 1 g of cells (wet weight) using the process according to the invention. The protein determination was carried out according to P.K. Smith et al., Anal. Biochem. Vol. 150, 76-85 (1985) using ovalbumin as a standard. The specific activity of the citrate lyase is ca. 70 U/ml protein (M. Single and P.A. Srere, J. Biol. Chem. Vol. 251 (10), 2911-2615 (1976)). The activity of the holo-enzyme that can be obtained by the process according to the invention is thus ca. 0.5 to 3-fold higher than the activity that was achieved with acetyl CoA and apo-citrate lyase.

Hence the process according to the invention provides for the first time a recombinant protein with improved citrate lyase activity that is both soluble and active.

Furthermore the invention concerns a test kit for the determination of citric acid which is composed

essentially of the following components: a protein obtainable by the process according to the invention with citrate lyase activity, at least one protein with hydrogen-transferring activity, nicotinamide-adenine dinucleotide or an appropriate derivative in a reduced form and optionally suitable stabilizers, activators and/or substances to avoid or reduce interferences i.e. components or reactions which mask or interfere with the actual reaction as well as suitable buffer solutions. In particular L-malate dehydrogenase and L-lactate dehydrogenase come into consideration as proteins with hydrogen-transferring activity. Those substances, additives or measures which help to avoid or at least to delay the degradation of a property or activity that is important for the determination are in principle suitable as stabilizers. Especially when only small amounts of sample material are available or if the samples are very dilute it can be advantageous to add activators.

An additional subject matter of the invention is the use of the recombinant soluble protein with citrate lyase activity to determine citric acid in clinical chemistry, food analysis and as a purity test for cosmetics. In clinical chemistry a corresponding enzymatic test is used primarily to examine fertility and for therapeutic monitoring of patients with kidney stones. In food analysis the most important application is analysis of wines and fruit juices.

The enzymatic method is based on the cleavage of citrate by the enzyme citrate lyase in the presence of Mg^{2+} ions to form oxaloacetate and acetate. In the presence of hydrogen-transferring enzymes such as L-malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate

and its decarboxylation product pyruvate are reduced by reduced NADH or NADPH to form L-malate and L-lactate. The amount of NADH or NADPH is proportional to the amount of citrate and is measured at 334 nm, 340 nm or 365 nm.

Hence the invention also concerns a corresponding test kit for the determination of citric acid which, apart from suitable buffer solutions, contains a recombinant protein with citrate lyase activity, one or several hydrogen-transferring enzymes and a nicotinamide adenine dinucleotide or a corresponding derivative in a reduced form and optionally suitable stabilizers such as thiol reagents.

Figure legends

Figure 1:
A: Function of the various subunits in a reaction catalysed by citrate lyase and activation of the enzyme by citrate lyase ligase. HS-R denotes a prosthetic group.

B. Structure of the prosthetic group of citrate lyase 2'-(5"-phosphoribosyl)-3'-phospho-CoA.

Figure 2:
Citrate lyase gene cluster from Klebsiella pneumoniae (K.p.), Escherichia coli (E.c.) Haemophilus influenzae (H.i.) and Leuconostoc mesenteroides (L.m.). Gene sequences that are homologous to E. coli citX are shown by the light grey shading.

INFORMATION FOR SEQ ID NO. 1:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 36 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- CCCTCTAGAGAACAACATTCGTTGCAAATCGATAAC - 3´

INFORMATION FOR SEQ ID NO. 2:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 38 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- CCGCGAATTCTTAGTTCCACATGGCGAGAATCGGCCAG -3´

INFORMATION FOR SEQ ID NO. 3:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 5484 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

```
   1  GAACAACATT CGTTGCAAAT CGATAACAAC ATGCACCTTC AGGATACTAT
         ┌Start citC
  51  TTATTATGTT CGGCAATGAT ATTTTCACCC GCGTAAAACG TTCAGAAAAT
 101  AAAAAAATGG CGGAAATCGC CCAATTCCTG CATGAAAATG ATTTGAGCGT
 151  TGACACCACA GTCGAAGTAT TTATTACCGT AACCCGCGAT GAAAAGCTTA
 201  TCGCGTGCGG TGGAATTGCC GGAAATATTA TTAAATGCGT TGCTATCAGT
 251  GAATCCGTCC GCGGTGAAGG ACTGGCGCTG ACATTAGCCA CTGAATTGAT
 301  AAACCTCGCC TATGAGCGGC ACAGCACGCA TCTGTTTATT TATACCAAAA
 351  CCGAATACGA GGCGCTGTTC CGCCAGTGCG GTTTTTCCAC GCTGACCAGC
 401  GTACCCGGCG TGATGGTGCT GATGGAAAAC AGCGCCACGC GACTGAAACG
 451  CTATGCCGAA TCGCTGAAAA AATTTCGTCA TCCAGGGAAC AAGATTGGCT
 501  GCATTGTGAT GAACGCCAAT CCCTTTACGA ATGGTCACCG TTATCTGATT
 551  CAACAGGCTG CGGCACAGTG CGACTGGTTG CATCTGTTTT TAGTCAAAGA
 601  AGATTCTTCA CGCTTCCCCT ATGAAGACCG GCTGGATTTG GTGTTAAAAG
 651  GCACCGCCGA TATTCCACGC CTGACTGTGC ATCGTGGCTC CGAATACATC
 701  ATCTCCCGCG CTACGTTCCC TTGCTACTTC ATTAAAGAAC AGAGCGTCAT
 751  TAACCATTGT TACACCGAAA TTGATCTGAA GATTTTCCGT CAGTACCTCG
 801  CTCCCGCGCT GGGTGTAACT CACCGCTTTG TCGGTACTGA ACCCTTTTGT
 851  CGCGTTACCG CCCAGTACAA CCAGGATATG CGCTACTGGC TGGAAACGCC
 901  GACTATCTCC GCACCGCCCA TCGAACTGGT TGAAATTGAG CGGCTGCGTT
```

```
951   ACCAGGAGAT GCCGATATCC GCTTCCCGGG TACGTCAACT GCTGGCGAAA
1001  AACGATCTCA CGGCTATCGC GCCGCTGGTC CCTGCAGTCA CGCTGCATTA
1051  TTTGCAGAAC CTGCTTGAGC ACTCCCGCCA GGACGCGGCA GCTCGTCAAA
           Stop citC┐                      ┌Start citD
1101  AGACCCCCGC ATGAGAAACA GGTGAAAAAT GAAAATAAAC CAGCCCGCCG
1151  TTGCAGGCAC CCTTGAGTCT GGGGATGTGA TGATACGCAT CGCCCCACTC
1201  GATACGCAGG ATATCGACCT GCAAATCAAT AGCAGCGTTG AGAAACAGTT
1251  TGGCGATGCA ATTCGCACCA CCATTCTGGA CGTTCTCGCC CGCTACAACG
1301  TGCGCGGCGT ACAGCTGAAT GTCGATGACA AAGGCGCACT GGACTGCATT
1351  TTACGTGCAC GACTGGAAGC CCTGCTGGCA CGCGCCAGCG GTATCCCGGC
                      Stop citD┐
1401  TCTGCCATGG GAGGATTGCC AATGATTTCC GCTTCGCTGC AACAACGTAA
                            └Start citE
1451  AACTCGCACC CGCCGCAGCA TGTTGTTTGT GCCTGGTGCC AATGCCGCGA
1501  TGGTCAGCAA CTCCTTCATC TACCCGGCTG ATGCCCTGAT GTTTGACCTC
1551  GAAGACTCCG TAGCATTGCG TGAAAAAGAC ACCGCCCGCC GCATGGTTTA
1601  CCACGCGCTG CAACATCCGC TGTATCGCGA TATTGAAACC ATTGTGCGTG
1651  TCAACGCGCT GGATTCCGAA TGGGGTGTTA ACGACCTGGA AGCCGTCGTT
1701  CGCGGTGGTG CGGACGTTGT GCGTCTGCCG AAAACCGATA CCGCTCAGGA
1751  TGTTCTGGAT ATTGAAAAAG AGATCCTGCG TATCGAAAAA GCCTGTGGTC
1801  GTGAACCCGG CAGCACCGGC CTGCTGGCGG CGATTGAATC TCCGCTGGGG
1851  ATTACCCGCG CAGTGGAAAT CGCTCACGCT TCCGAGCGTT TGATCGGTAT
1901  CGCCCTCGGT GCAGAAGACT ATGTGCGCAA CCTGCGTACA GAACGCTCCC
1951  CGGAAGGAAC TGAACTGCTG TTCGCACGCT GTTCCATTTT GCAGGCCGCG
2001  CGCTCTGCGG GTATTCAGGC GTTCGATACC GTCTATTCCG ACGCTAACAA
2051  CGAAGCCGGA TTTCTGCAAG AAGCCGCCCA CATCAAACAG CTGGGCTTTG
2101  ACGGCAAATC GCTGATCAAC CCGCGTCAGA TTGATCTGCT GCACAACCTC
2151  TACGCACCGA CCCAGAAAGA AGTGGATCAC GCCCGCCGCG TCGTAGAAGC
2201  CGCTGAAGCC GCCGCTCGCG AAGGCCTCGG CGTGGTTTCC CTGAACGGCA
2251  AGATGGTGGA CGGTCCGGTT ATCGATCGCG CCCGTCTGGT GCTCTCCCGT
                          Stop citE┐                ┌Start citF
2301  GCAGAACTTT CCGGCATCCG CGAAGAATAA GGCAATCAAA ATGACGCAGA
2351  AAATTGAACA ATCTCAACGA CAAGAACGGG TAGCGGCCTG GAATCGTCGC
2401  GCTGAATGCG ATCTTGCCGC TTTCCAGAAC TCGCCAAAGC AAACCTACCA
2451  GGCTGAAAAA GCGCGCGATC GCAAACTGTG CGCCAACCTG GAAGAAGCGA
2501  TTCGTCGCTC TGGTTTACAG GACGGCATGA CGGTTTCCTT CCATCACGCT
2551  TTCCGTGGCG GTGACCTGAC CGTCAATATG GTGATGGACG TCATCGCGAA
2601  GATGGGCTTT AAAAACCTGA CCCTGGCGTC CAGCTCCCTG AGTGATTGCC
2651  ATGCGCCGCT GGTAGAACAC ATTCGCCAGG GCGTGGTTAC CCGCATTTAT
2701  ACCTCCGGCC TGCGTGGTCC ACTGGCGGAA GAGATCTCCC GTGGTCTGCT
2751  GGCAGAACCG GTGCAGATCC ACTCTCACGG CGGTCGTGTG CATCTGGTAC
2801  AGAGCGGCGA ACTGAATATC GACGTGGCTT TCCTCGGCGT CCCGTCCTGT
2851  GATGAATTCG GTAATGCCAA CGGCTACACC GGTAAAGCCT GCTGCGGCTC
2901  CCTCGGCTAT GCAATAGTTG ATGCCGACAA CGCAAAACAG GTCGTGATGC
2951  TTACCGAAGA ACTGCTGCCT TATCCGCATA ATCCGGCAAG CATTGAGCAA
3001  GATCAGGTTG ATTTGATCGT CAAAGTTGAC CGCGTTGGCG ATGCTGCAAA
3051  AATCGGCGCT GGCGCGACCC GTATGACCAC TAACCCGCGC GAACTGCTTA
3101  TTGCCCGTAG CGCTGCGGAT GTGATTGTCA ACTCTGGCTA CTTCAAAGAA
3151  GGTTTCTCCA TGCAAACCGG CACCGGCGGC GCATCGCTGG CGGTAACCCG
3201  TTTCCTGGAA GACAAAATGC GTAGCCGCGA TATTCGCGCC GACTTCGCCC
3251  TTGGCGGTAT TACCGCGACG ATGGTTGACC TGCACGAAAA AGGTCTGATC
3301  CGCAAACTGC TGGATGTGCA GAGCTTTGAC AGCCATGCTG CGCAATCGCT
3351  GGCCCGTAAC CCCAATCACA TCGAAATCAG CGCCAACCAG TACGCTAACT
```

```
3401   GGGGTTCGAA AGGCGCATCG GTTGATCGTC TCGACGTGGT GGTACTGAGC
3451   GCGCTGGAAA TTGACACCCA GTTCAACGTT AACGTGCTGA CCGGCTCTGA
3501   CGGCGTACTG CGTGGTGCTT CCGGTGGTCA CTGCGATACC GCGATTGCCT
3551   CTGCGCTTTC CATCATCGTC GCGCCGCTGG TACGCGGTCG TATTCCGACT
3601   CTGGTGGATA ACGTACTGAC CTGCATCACC CCAGGCTCCA GTGTCGATAT
3651   TCTGGTCACA GACCACGGTA TCGCAGTTAA CCCGGCACGT CCGGAACTGG
3701   CAGAACGTCT GCAGGAAGCG GGCATTAAAG TGGTTTCCAT TGAGTGGCTG
3751   CGCGAACGTG CGCGTCTGCT GACCGGTGAA CCACAGCCGA TTGAATTCAC
3801   AGACCGCGTC GTTGCCGTTG TGCGTTACCG CGATGGCTCG GTGATCGATG
                  Stop citF┐        ┌Start citX
3851   TTGTGCATCA GGTGAAGGAA TAAGCCATGC ACCTGCTTCC TGAACTCGCC
3901   AGCCACCATG CGGTATCAAT TCCCGAGCTG CTCGTCAGCC GGGATGAAAG
3951   GCAAGCACGG CAACACGTCT GGCTCAAGCG CCATCCTGTT CCACTGGTCT
4001   CCTTTACCGT GGTTGCGCCT GGGCCGATTA AAGACAGCGA GGTCACACGC
4051   CGAATTTTTA ATCATGGCGT GACAGCCTTG CGTGCCTTAG CCGCAAAACA
4101   GGGCTGGCAA ATTCAGGAGC AGGCTGCACT GGTTTCCGCC AGCGGGCCGG
4151   AGGGCATGTT GAGCATTGCC GCCCCGGCTC GCGACCTCAA GCTCGCCACC
4201   ATTGAGCTTG AACATAGTCA TCCTCTCGGG CGGTTATGGG ATATCGATGT
4251   CCTGACGCCC GAAGGCGAAA TTCTCTCCCG CCGCGACTAT TCACTGCCGC
4301   CTCGCCGCTG CCTGTTGTGC GAACAAAGCG CAGCCGTCTG CGCGCGTGGA
4351   AAAACCCATC AACTGACCGA TTTACTCAAC CGCATGGAGG CACTGCTGAA
                             Stop citX┐
4401   CGATGTCGAT GCCTGCAACG TCAACTAAAA CCACAAAGCT TGCGACGTCA
        └Start citG
4451   TTAATCGATG AGTACGCCCT GCTGGGCTGG CGCGCCATGC TGACTGAAGT
4501   CAATCTGTCA CCGAAACCAG GCCTCGTGGA TCGCATTAAC TGCGGTGCGC
4551   ACAAAGATAT GGCGCTGGAA GATTTCCACC GCAGCGCGCT GGCGATTCAG
4601   GGCTGGCTAC CCCGTTTCAT TGAATTTGGT GCCTGTAGTG CGGAAATGGC
4651   ACCAGAAGCG GTACTCCACG GATTACGCCC AATTGGTATG GCTTGCGAAG
4701   GTGATATGTT CCGCGCCACT GCGGGCGTAA ACACGCATAA AGGCAGCATT
4751   TTTTCTTTAG GGCTGCTATG TGCGGCAATT GGCCGTTTGC TTCAACTCAA
4801   CCAACCGGTA ACGCCAACAA CCGTTTGTTC TACGGCGGCA AGTTTCTGCC
4851   GTGGCCTGAC CGATCGCGAA CTGCGTACCA ATAATTCACA ACTGACGGCA
4901   GGTCAACGGT TGTACCAACA GCTTGGCCTT ACCGGCGCAC GCGGTGAAGC
4951   CGAAGCGGGT TATCCACTGG TGATCAATCA CGCCTTGCCG CATTACCTCA
5001   CTCTGCTGGA TCAGGGGTTA GATCCTGAAC TGGCATTGCT CGATACCTTG
5051   CTCCTACTGA TGGCGATCAA CGGCGATACC AACGTTGCAT CGCGCGGTGG
5101   CGAGGGGGGC CTGCGCTGGC TACAGCGCGA GGCGCAAACA TTATTGCAAA
5151   AAGGGGGCAT TCGAACCCCC GCCGATCTCG ATTATCTCCG GCAGTTCGAC
5201   AGGGAGTGTA TCGAACGAAA TCTCAGTCCA GGCGGCAGTG CTGACCTACT
                             Stop citG┐
5251   GATCCTTACC TGGTTTTTAG CACAGATTTA ATTATTTAAG CACTTGATAA
                                         ┌Start citT
5301   ATTTGGAAAT ATTAATTTTC GGAGAACCCG TATGTCTTTA GCAAAAGATA
5351   ATATATGGAA ACTATTGGCC CCACTGGTGG TGATGGGTGT CATGTTTCTT
5401   ATCCCTGTCC CCGACGGTAT GCCGCCGCAG GCATGGCATT ACTTCGCTGT
5451   GTTTGTGGCA ATGATTGTCG GCATGATCCT CGAG
```

INFORMATION FOR SEQ ID NO. 4:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 33 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- AAATTTCATATGCACCTGCTTCCTGAACTCGCC - 3´

INFORMATION FOR SEQ ID NO. 5:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 36 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- GGGCCCCTCGAGTTAGTTGACGTTGCAGGCATCGAC - 3´

INFORMATION FOR SEQ ID NO. 6:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 553 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

```
  1  ATGCACCTGC TTCCTGAACT CGCCAGCCAC CATGCGGTAT CAATTCCCGA
 51  GCTGCTCGTC AGCCGGGATG AAAGGCAAGC ACGGCAACAC GTCTGGCTCA
101  AGCGCCATCC TGTTCCACTG GTCTCCTTTA CCGTGGTTGC GCCTGGGCCG
151  ATTAAAGACA GCGAGGTCAC ACGCCGAATT TTTAATCATG GCGTGACAGC
201  CTTGCGTGCC TTAGCCGCAA AACAGGGCTG GCAAATTCAG GAGCAGGCTG
251  CACTGGTTTC CGCCAGCGGG CCGGAGGGCA TGTTGAGCAT TGCCGCCCCG
301  GCTCGCGACC TCAAGCTCGC CACCATTGAG CTTGAACATA GTCATCCTCT
351  CGGGCGGTTA TGGGATATCG ATGTCCTGAC GCCCGAAGGC GAAATTCTCT
401  CCCGCCGCGA CTATTCACTG CCGCCTCGCC GCTGCCTGTT GTGCGAACAA
451  AGCGCAGCCG TCTGCGCGCG TGGAAAAACC CATCAACTGA CCGATTTACT
501  CAACCGCATG GAGGCACTGC TGAACGATGT CGATGCCTGC AACGTCAACT
551  AA
```

INFORMATION FOR SEQ ID NO. 7:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 5593 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

```
   1 TTAATTAACA ACATAAAAAC CATAAAGCCA ATTAAGCCAC GAGAAAAACT GTGACTTAAA
  61 TACAAGAATC CATAGCCGAA CGCTGGCGAA ATACAGTTCG TTTTGAAATG ACGAAGCGCT
        ┌Start citCe
 121 AAAAAATGAC ACTGATATTA AAACGCGTTC AGCTATTAAA AGATAAACCG CGGCGAGAGG
 181 CGATCGATCG GTTTCTCCGC CAGCATCAAC TGTCGTTAGA GGCCGACTGC GAAATGGCGA
 241 TTATCGCCGA GTATCAGCAG CGGCTGGTCG GCTGCGGTGC TATCGCCGGC AATGTGCTGA
 301 AATGCATCGC CATCGATCCC TCGCTGCAGG GGGAGGGGCT GAGCCTTAAA TTACTGACCG
 361 AGCTCCTGAC GCTGGCCTAT GAGCTGGGGC GCAGCGAACT GTTTTTGTTC ACTAAACCTT
 421 GCAATGCCGC GTTATTTTCC GGCGCCGGCT TCTGGCCGAT AGCCCAGGCG GGCGACCGCG
 481 CCGTGCTAAT GGAAAATAGC CGCGAACGGC TGACTCGTTA CTGTCGACAG CTGGCGATGT
 541 ACCGTCAGCC GGGAAGAAAA ATCGGCGCTA TCGTGATGAA TGCTAATCCA TTCACCCTCG
 601 GCCACCGCTG GTTGGTAGAA CAGGCGGCCA GCCAGTGCGA CTGGCTGCAT CTGTTTGTGG
 661 TCAAAGAAGA TGCGTCCTGC TTTTCCTATC ACGATCGCTT CAAGCTCATT GAACAGGGGA
 721 TTACCGGCAT CGATAAGGTG ACGCTGCATC CCGGTTCGGC GTATCTGATC TCGCGGGCGA
 781 CGTTCCCCGG CTATTTCCTG AAAGAGCAGG GGGTGGTTGA TGACTGCCAC AGCCAGATTG
 841 ACCTGCAGCT CTTCCGCGAG CGCCTGGCCC CGGCGCTGCA GATTACCCAT CGCTTTGTCG
 901 GCACCGAGCC GCTGTGTCCC CTGACCCGTA ATTACAACCA GCGCATGAAG TCACTACTGG
 961 AAGCGCCAGG CGACGCGCCG CCCATTGAAG TAGTTGAGCT TGCGCGAATC GAAAAAAATG
1021 GTGGACCCGT GTCGGCCTCC CGAGTGCGCG AACTCTATCG ACAGCGCAAC TGGCAGGCGG
1081 TCGCGGCGCT GGTACCGCCG GGAACCCTCT CTTTTCTGAT GCAACTGGCG GAAAGCGAAC
       Stop citC┐                                    ┌Start citD
1141 ATCAAACCGC CTGATTTATA CGCCCTAACT AAGGATTTTC CCCTATGGAA ATGAAGATTG
1201 ACGCCCTGGC CGGCACGCTG GAGTCCAGCG ATGTGATGGT CAGGATTGGA CCCGCGGCGC
1261 AGCCGGGCAT TCAGCTGGAA ATCGACAGCA TTGTGAAACA ACAGTTTGGC GCTGCGATTG
1321 AGCAGGTAGT GAGAGAAACG CTGGCTCAGC TTGGCGTGAA ACAGGCCAAC GTGGTGGTCG
1381 ATGATAAAGG CGCGCTGGAA TGTGTTTTGC GAGCTCGCGT ACAGGCCGCG GCGCTGCGCG
                                  Stop citD┐
1441 CGGCGCAACA GACCCAATTA CAATGGAGCC AGCTATGAAA CCACGTCGCA GTATGTTGTT
                                           └Start citE
1501 CATCCCTGGC GCCAATGCCG CCATGTTAAG CACGTCATTC GTCTACGGCG CTGATGCTGT
1561 GATGTTCGAC CTGGAAGATG CCGTTTCGCT GCGCGAGAAA GATACCGCTC GTCTGCTGGT
1621 GTATCAGGCG CTGCAGCATC CACTGTATCA GGATATCGAA ACCGTGGTGC GTATTAACCC
1681 GCTAAATACC CCGTTTGGTC TGGCCGATCT GGAAGCCGTG GTTCGTGCGG GCGTGGATAT
1741 GGTGCGTCTG CCGAAAACCG ACAGCAAAGA AGATATCCAT GAGCTGGAAG CGCATGTTGA
1801 GCGGATTGAA CGCGAGTGCG GCCGGGAAGT GGGCAGCACC AAGTTAATGG CGGCGATCGA
1861 GTCGGCGCTG GGCGTGGTGA ACGCGGTGGA AATCGCCCGC GCCAGCCCGC GTCTGGCGGC
1921 GATCGCGCTG GCGGCCTTCG ATTACGTGAT GGATATGGGC ACCTCCCGCG GCGACGGTAC
1981 TGAACTGTTC TACGCCCGCT GCGCTGTACT GCATGCCGCC CGCGTTGCCG GCATCGCCGC
2041 CTATGACGTG GTGTGGTCGG ATATCAATAA TGAAGAGGGC TTCCTGGCGG AAGCGAATCT
2101 GGCCAAAAAC CTCGGCTTTA ACGGCAAATC GTTGGTTAAC CCACGACAAA TTGAACTCCT
2161 GCATCAGGTC TATGCCCCGA CGCGCAAAGA GGTCGATCAC GCGCTGGAAG TGATTGCCGC
2221 GGCGGAAGAA GCCGAAACGC GAGGTCTGGG TGTGGTATCG CTGAACGGCA AGATGATCGA
2281 TGGACCGATT ATCGACCATG CTCGCAAAGT GGTGGCGCTC TCGGCTTCCG GTATTCGTGA
```

Stop citE Start citF

2341 TTAAGGGGAA TAAGATGAAA GAGACAGTAG CAATGCTTAA TCAGCAGTAC GTGATGCCGA
2401 ATGGACTGAC ACCTTATGCC GGCGTAACGG CGAAAAGTCC CTGGCTGGCG AGTGAGAGCG
2461 AAAAGCGCCA GCGCAAAATC TGCGATTCGC TGGAAACGGC AATCCGTCGC TCCGGCCTGC
2521 AAAACGGCAT GACCATCTCG TTTCACCACG CGTTTCGCGG CGGTGACAAA GTCGTCAATA
2581 TGGTAGTGGC GAAGCTGGCG GAAATGGGTT TTCGCGATCT CACCCTGGCG TCCAGTTCGC
2641 TGATCGACGC CCACTGGCCG CTGATCGAGC ATATTAAAAA TGGCGTGATC CGCCAGATCT
2701 ACACCTCCGG CCTGCGCGGC AAGTTGGGCG AGGAGATCTC CGCCGGTTTA ATGGAAAACC
2761 CGGTGCAGAT CCACTCCCAC GGCGGTCGCG TACAGCTGAT TCAAAGCGGC GAGCTGTCGA
2821 TTGATGTCGC GTTTCTCGGC GTTCCTTGCT GCGATGAGTT TGGCAACGCC AACGGCTTTA
2881 GCGGTAAATC ACGCTGCGGT TCTCTGGGCT ACGCGCGCGT CGATGCCGAG CACGCTAAAT
2941 GCGTGGTGCT GCTCACCGAA GAGTGGGTGG ATTATCCTAA CTATCCGGCC AGTATTGCCC
3001 AGGATCAGGT GGATCTGATA GTCCAGGTAG ATGAAGTCGG CGATCCGCAA AAAATTACCG
3061 CGGGTGCCAT CCGTCTGACC AGCAACCCGC GCGAGCTGCT GATCGCCCGC CAGGCGGCGA
3121 AAGTCGTTGA GCACTCCGGT TACTTTAAAG AGGGTTTCTC GCTGCAGACC GGTACCGGCG
3181 GCGCCTCGCT GGCAGTAACT CGCTTCCTTG AAGATAAAAT GCGCCGTAAC GGCATTACCG
3241 CCAGCTTCGG CCTCGGCGGT ATCACCGGGA CGATGGTCGA TTTGCACGAA AAAGGGTTGA
3301 TCAAAACGCT GCTCGATACC CAGTCCTTCG ATGGTGACGC GGCGCGTTCG CTGGCGCAGA
3361 ACCCGAACCA TGTCGAGATC TCCACCAATC AGTATGCCAG CCCGGGCTCC AAAGGCGCCT
3421 CCTGCGAGCG CTTAAACGTG GTGATGCTCA GCGCGCTGGA AATTGATATC GACTTTAACG
3481 TTAACGTGAT GACCGGTTCT AACGGTGTGC TGCGCGGGGC GTCCGGTGGC CATAGCGATA
3541 CCGCCGCCGG TGCGGATTTG ACCATTATTA CCGCGCCGTT AGTTCGCGGC CGTATTCCCT
3601 GCGTCGTGGA AAAGGTGCTG ACCCGCGTCA CGCCGGGGGC CAGCGTGGAT GTGCTGGTCA
3661 CTGACCACGG CATTGCGGTC AACCCGGCAC GTCAGGACCT GATCGACAAT TTGCGCAGCG
3721 CAGGCATTCC GCTGATGACC ATTGAGGAAC TGCAGCAGCG TGCTGAGCTG TTGACTGGCA
3781 AGCCGCAGCC GATCGAATTC ACCGATCGGG TGGTGGCGGT GGTGCGCTAT CGCGACGGTT

Stop citF

Start

3841 CGGTCATCGA TGTGATTCGT CAGGTGAAAA ACAGCGACTA AACGCAGAGG GGAAAGGCCA

citG

3901 TGAGCGACGT GTTAATTAAT CCTGCGCGTG TGCGGCGCGT GAAGCCACTG AGTGCCGAAG
3961 AGGTGGTCAG CGCGGTAGAG CGCGCGCTGT TGACCGAAGT TCGCCTGACC CCAAAGCCCG
4021 GGTTGGTGGA TATTCGTAAC GCTGGCGCGC ACTGGGATAT GGATCTGGCC TCGTTTGAGG
4081 CCAGCACCGC GGTGGTGGCT CCGTGGATGG AGAAATTTTT CATCATGGGC CACGATACTG
4141 CGGCGGTCGC GCCGGAGCAG GTATTGATGA TGCTGCGCCC GGTAGGGATG GCCTGTGAGA
4201 ACGATATGCT GGAGGCCACC GGCGGGGTGA ATACCCATCG CGGGGCGATC TTCGCTTTTG
4261 GCCTGCTCAG CGCGGCGGCG GGCAGGCTGG TGTCGAAAGG TGAGCCGATA GAGCAGCACC
4321 GGCTTTGCGA CCAGGTGGCG CGCTTCTGTC GCGGCATGGT TATGCAGGAG TTGTCTTCTG
4381 CTGGCGGGGA ACGGCTCAGT AAAGGCGAGG CTCATTTTCT ACGCTATGGT CTCTCCGGGG
4441 CCCGCGGCGA GGCGGAGAGC GGTTTCCTGA CGGTGCGTAC CCAGGCCATG CCAGTCTTTA
4501 CCCGCATGAT GGAAGAGACC GGCGACAGTA ATCTGGCGCT ACTGCAAACC CTGCTGCATC
4561 TGATGGCGTG GAATGATGAC ACCAACCTGG TCTCGCGCGG CGGGCTTGCC GGGCTGAACT
4621 TTGTCCAGCA GGAGGCGCAG CGACTGCTGT GGCAGGGCGG CGTGCTGGCG GACGGCGGGC
4681 TGGAGGCGCT GCGACAGTTT GACGATGAGC TGATTGCCCG CCATCTCAGC CCTGGCGGCA
4741 GCGCCGATCT GTTGGCGGTG ACCTGGTTTT TATCCGCGTT TCCCGCCGGC GCGCTTTTCC

Stop citG

4801 CGCTGTAACC CACTGCAATA CCGCCTTCGC CCGCACTGTA CGGGCGAGGG CGCCATCATT
4861 AGCCTTCCCG GTTGTCATCC GGTAAACACG GAATCGCGGC ACAATCGTAT AGTTTTTACT
4921 GATATCGTCC GCCGTTTGTC ATAAATTTCT AATTATCGGC GTTTTTGAGT AGCGGCCCGC
4981 TGACGGGCTG GTTACTCTGA AAACAATTTA CGTAATGTTA ACAAAAGAGA ATAGCTATGC
5041 ATGATGCACA AATCCGCGTG GCCATCGCCG GCGCGGGCGG CCGGATGGGA CGCCAGTTAA
5101 TTCAGGCTGC ATTGCAGATG GAAGGCGTGG CGCTGGGCGC GGCGCTGGAG CGCGAAGGGT
5161 CAAGCCTGGT GGGCAGCGAC GCCGGCGAGC TGGCGGGCGC CGGCAAAGCG GGCGTCGCGG

```
5221 TGCAGAGCAG CCTGGCGGCG GTAAAAGATG ATTTCGACGT GTTGATCGAT TTTACCCGCC
5281 CGGAAGGCAC GCTGAACCAT CTGGCGTTTT GCCGCGAGCA CGGCAAAGGG ATGGTCATCG
5341 GCACCACCGG TTTTGACGAC GCTGGCAAAC AGGCGATTCG CGATGCCGCG CAGGACATTG
5401 CCATTGTCTT CGCCGCTAAC TTTAGCGTTG GCGTCAATGT CCTGTTGAAG CTGCTGGAGA
5461 AGGCGGCGAA GGTGATGGGC GACTATACCG ACATCGAAAT TATCGAAGCG CACCACCGGC
5521 ATAAAGTGGA TGCGCCGTCA GGCACCGCGC TGGCGATGGG CGAAGCGATC GCCGGGGCAT
5581 TGAACAAAGA TCT
```

The invention is further elucidated by the following examples:

Example 1:

Cell culture

The following strains and plasmids were used: E. coli DH5α or BL21 (DE3) (F.W. Studiar and B.A. Mofatt, J. Mol. Biol. Vol. 189, 113-130 (1986)) and pACYC184 (A.C.Y. Chang et al., J. Bacteriol. Vol. 134, 1141-1156 (1978)). The E. coli cells were routinely cultured in Luria Bertani (LB) medium at 37°C according to J. Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2nd Edition 1989). Antibiotics were added at the following final concentrations: 200 µg/ml ampicillin, 50 µg/ml chloroamphenicol and 50 µg/ml kanamycin. The E. coli strain DH5α was used as the host organism for the cloning. The E. coli BL21 (DE3) cells which contain the phage T7 polymerase gene under the control of a lacUV5 promoter (F.W.Studier and B.A. Moffatt, supra) served as a host for the expression of the target genes of pT7-7 and pET derivatives. The cultures for the expression were prepared as follows. After centrifugation (3000 g, 8 min) of a preculture of 40 ml which had been incubated overnight at 37°C, the cells were resuspended in 20 ml fresh LB medium. The cell suspension was subsequently

used to inoculate 2 L of the same medium which contained appropriate antibiotics and the culture was incubated at 37°C in a shaker (180 rpm). When the OD_{600} reached a value between 0.5 and 0.8, the expression of the target genes was induced by adding IPTG (isopropyl-β-D-thiogalactoside) at a final concentration of 1 mM and the culture was incubated for a further 3 hours at 37°C in a shaker (180 rpm). Subsequently the cells were harvested by centrifugation (30 min at 3000 g), washed once with 20 ml 50 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$ and stored at -20°C.

Example 2:

Isolation of the genes and gene cluster

For the construction of the expression plasmid which contains the E. coli citCDEFXG gene cluster, a 6.9 kb fragment from the chromosomal DNA of E. coli was amplified by means of PCR with the primers eccl-for (SEQ ID NO.1) and ec-citT-rev (SEQ ID NO.2) using the Expand High Fidelity PCR System from Roche Diagnostics. The 6.9 kb PCR fragment which additionally contains the citT gene (K.M. Pos et al., J. Bacteriol. Vol. 180, 4160-4165 (1998)), was cleaved with the restriction endonucleases XbaI and XhoI and the resulting 5.5 kb fragment (SEQ ID NO.3) and an expression vector that was also linearized correspondingly such as pKK177-3Hb, pKKT5, pUC18, pT7, pET24b were separated on an agarose gel and the appropriate bands were isolated (QIAEX kit from the Diagen Company). Subsequently the PCR fragment and the vector fragment were ligated together using T4 DNA ligase. For this 1 µl (20 ng) vector fragment and 3 µl (100 ng) PCR fragment, 1 µl 10 x ligase buffer (Maniatis et al., 1989 B.27), 1 µl T4 DNA ligase, 4 µl sterile redistilled H_2O were pipetted, carefully mixed

and incubated overnight at 16°C. The insert obtained from the PCR starts 55 bp before the citC start codon and ends 203 bp downstream of the citG stop codon.

For the construction of the expression plasmid which contains the citX gene from E. coli (SEQ ID NO.3), the citX gene was amplified by PCR from the chromosomal DNA with the primers ec-citX-for (SEQ ID NO.4) and ec-citX-rev (SEQ ID NO.5) using the Pfu DNA polymerase (Stratagene). The start codon is part of an NdeI restriction endonuclease cleavage site and a XhoI restriction endonuclease cleavage site is located directly behind the stop codon. After digestion of the PCR product with NdeI and XhoI, the resulting 555 bp DNA fragment (SEQ ID NO.6) was ligated into appropriately linearized expression vectors (as described above).

The construction of the expression plasmid which contains the citCDEFG gene cluster of Klebsiella pneumoniae is described in M. Bott and P. Dimroth, Molecular Microbiology Vol. 14 (2), 347-356 (1994). The sequence of the citCDEFG gene cluster is shown in SEQ ID NO.7.

Example 3:

Transformation of the various expression plasmids in various E. coli expression strains

Competent cells of various E. coli strains were prepared according to the method of Hanahan (J. Mol. Biol. Vol. 166, 557 ff. (1983)). 200 µl of cells prepared in this manner were mixed with 20 ng of the corresponding expression plasmids. After 30 minutes incubation on ice, a heat shock was carried out (90 sec. at 42°C).

Subsequently the cells were transferred to 1 ml LB medium and incubated for 1 hour at 37°C for the phenotypic expression. Aliquots of this transformation mixture were plated on LB plates containing the appropriate antibiotic as a selection marker and incubated for 15 hours at 37°C.

Example 4:

Expression of the various target genes

After centrifugation (3000 g, 8 min) of 40 ml preculture which had been grown overnight at 37°C, the cell pellet was resuspended in 20 ml fresh LB medium. The cell suspension was then used to inoculate 2 l LB medium containing the appropriate antibiotics. This cell culture was incubated at 37°C in a shaker (180 rpm). The expression of the target genes was induced at an optical density (measured at 600 nm) of 0.5 - 0.8 by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG, final concentration) and the cultures were incubated for a further 3 hours at 37°C and 180 rpm. Afterwards the cells were harvested by centrifugation (30 min. at 3000 g), washed once in 20 ml 50 mM potassium phosphate, pH 7.0 and frozen at -20°C.

For the cell extract preparation, 1 g cells (wet weight) were resuspended in 4 ml cold 50 mM potassium phosphate, 1 mM $MgCl_2$ pH 7.0. After adding a protease inhibitor cocktail (Roche Diagnostics) and DNAseI to a final concentration of 25 mg/ml, the cells were lysed by a three-fold passage in a French press at 108 Mpa. Intact cells and cell debris were removed by centrifugation (30 min. at 27,000 g). The cell-free supernatant was separated from the membrane fraction by ultracentrifugation (1 H at 150,000 g) and the resulting

cell extract can then be used directly for enzymatic studies and for protein purification.

Example 5:

Citrate lyase activity test

The citrate lyase activity was measured at 25°C in a spectrophotometric test coupled with malate dehydrogenase from Roche Diagnostics. The test mixture contained in a final volume of 1 ml 50 mM glycylglycine pH 7.9, 5 mM potassium citrate, 2 mM $ZnCl_2$, 0.5 mM NADH, 30 U malate dehydrogenase (Roche Diagnostics) and 10 µl or 20 µl cell extract. The oxidation of NADH was measured in a spectrophotometer at 365 nm (ε = 3.4 mM^{-1} cm^{-1}). One enzyme unit (unit) is defined as 1 µmol citrate which is degraded per minute to acetate and oxaloacetate.

SEQUENCE LISTING

<110> Roche Diagnostics GmbH

<120> Process for the recombinant production of holo-citrate lyase

<130> 523400EP

<140>
<141>

<160> 7

<170> PatentIn Ver. 2.1

<210> 1
<211> 36
<212> DNA
<213> E. coli

<400> 1
ccctctagag aacaacattc gttgcaaatc gataac 36

<210> 2
<211> 38
<212> DNA
<213> E. coli

<400> 2
ccgcgaattc ttagttccac atggcgagaa tcggccag 38

<210> 3
<211> 5484
<212> DNA
<213> E. coli

<400> 3
gaacaacatt cgttgcaaat cgataacaac atgcaccttc aggatactat ttattatgtt 60
cggcaatgat attttcaccc gcgtaaaacg ttcagaaaat aaaaaaatgg cggaaatcgc 120
ccaattcctg catgaaaatg atttgagcgt tgacaccaca gtcgaagtat ttattaccgt 180
aacccgcgat gaaaagctta tcgcgtgcgg tggaattgcc ggaaatatta ttaaatgcgt 240
tgctatcagt gaatccgtcc gcggtgaagg actggcgctg acattagcca ctgaattgat 300
aaacctcgcc tatgagcggc acagcacgca tctgtttatt tataccaaaa ccgaatacga 360
ggcgctgttc cgccagtgcg gtttttccac gctgaccagc gtacccggcg tgatggtgct 420
gatggaaaac agcgccacgc gactgaaacg ctatgccgaa tcgctgaaaa aatttcgtca 480
tccagggaac aagattggct gcattgtgat gaacgccaat ccctttacga atggtcaccg 540
ttatctgatt caacaggctg cggcacagtg cgactggttg catctgtttt tagtcaaaga 600
agattcttca cgcttccccc atgaagaccg gctggatttg gtgttaaaag gcaccgccga 660
tattccacgc ctgactgtgc atcgtggctc cgaatacatc atctcccgcg ctacgttccc 720
ttgctacttc attaaagaac agagcgtcat taaccattgt tacaccgaaa ttgatctgaa 780
gattttccgt cagtacctcg ctcccgcgct gggtgtaact caccgctttg tcggtactga 840
acccttttgt cgcgttaccg cccagtacaa ccaggatatg cgctactggc tggaaacgcc 900
gactatctcc gcaccgccca tcgaactggt tgaaattgag cggctgcgtt accaggagat 960

```
gccgatatcc gcttcccggg tacgtcaact gctggcgaaa aacgatctca cggctatcgc 1020
gccgctggtc cctgcagtca cgctgcatta tttgcagaac ctgcttgagc actcccgcca 1080
ggacgcggca gctcgtcaaa agaccccgc atgagaaaca ggtgaaaaat gaaaataaac 1140
cagcccgccg ttgcaggcac ccttgagtct ggggatgtga tgatacgcat cgccccactc 1200
gatacgcagg atatcgacct gcaaatcaat agcagcgttg agaaacagtt tggcgatgca 1260
attcgcacca ccattctgga cgttctcgcc cgctacaacg tgcgcggcgt acagctgaat 1320
gtcgatgaca aaggcgcact ggactgcatt ttacgtgcac gactggaagc cctgctggca 1380
cgcgccagcg gtatcccggc tctgccatgg gaggattgcc aatgatttcc gcttcgctgc 1440
aacaacgtaa aactcgcacc cgccgcagca tgttgtttgt gcctggtgcc aatgccgcga 1500
tggtcagcaa ctccttcatc tacccggctg atgccctgat gtttgacctc gaagactccg 1560
tagcattgcg tgaaaaagac accgcccgcc gcatggttta ccacgcgctg caacatccgc 1620
tgtatcgcga tattgaaacc attgtgcgtg tcaacgcgct ggattccgaa tggggtgtta 1680
acgacctgga agccgtcgtt cgcggtggtg cggacgttgt gcgtctgccg aaaaccgata 1740
ccgctcagga tgttctggat attgaaaaag agatcctgcg tatcgaaaaa gcctgtggtc 1800
gtgaacccgg cagcaccggc ctgctggcgg cgattgaatc tccgctgggg attacccgcg 1860
cagtggaaat cgctcacgct tccgagcgtt tgatcggtat cgccctcggt gcagaagact 1920
atgtgcgcaa cctgcgtaca gaacgctccc cggaaggaac tgaactgctg ttcgcacgct 1980
gttccatttt gcaggccgcg cgctctgcgg gtattcaggc gttcgatacc gtctattccg 2040
acgctaacaa cgaagccgga tttctgcaag aagccgccca catcaaacag ctgggctttg 2100
acggcaaatc gctgatcaac ccgcgtcaga ttgatctgct gcacaacctc tacgcaccga 2160
cccagaaaga agtggatcac gcccgccgcg tcgtagaagc cgctgaagcc gccgctcgcg 2220
aaggcctcgg cgtggtttcc ctgaacggca agatggtgga cggtccggtt atcgatcgcg 2280
cccgtctggt gctctcccgt gcagaacttt ccggcatccg cgaagaataa ggcaatcaaa 2340
atgacgcaga aaattgaaca atctcaacga caagaacggg tagcggcctg gaatcgtcgc 2400
gctgaatgcg atcttgccgc tttccagaac tcgccaaagc aaacctacca ggctgaaaaa 2460
gcgcgcgatc gcaaactgtg cgccaacctg gaagaagcga ttcgtcgctc tggtttacag 2520
gacggcatga cggtttcctt ccatcacgct ttccgtggcg gtgacctgac cgtcaatatg 2580
gtgatggacg tcatcgcgaa gatgggcttt aaaaacctga ccctggcgtc cagctccctg 2640
agtgattgcc atgcgccgct ggtagaacac attcgccagg gcgtggttac ccgcatttat 2700
acctccggcc tgcgtggtcc actggcggaa gagatctccc gtggtctgct ggcagaaccg 2760
gtgcagatcc actctcacgg cggtcgtgtg catctggtac agagcggcga actgaatatc 2820
gacgtggctt tcctcggcgt cccgtcctgt gatgaattcg gtaatgccaa cggctacacc 2880
ggtaaagcct gctgcggctc cctcggctat gcaatagttg atgccgacaa cgcaaaacag 2940
gtcgtgatgc ttaccgaaga actgctgcct tatccgcata atccggcaag cattgagcaa 3000
gatcaggttg atttgatcgt caaagttgac cgcgttggcg atgctgcaaa aatcggcgct 3060
ggcgcgaccc gtatgaccac taacccgcgc gaactgctta ttgcccgtag cgctgcggat 3120
gtgattgtca actctggcta cttcaaagaa ggtttctcca tgcaaaccgg caccggcggc 3180
gcatcgctgg cggtaacccg tttcctggaa gacaaaatgc gtagccgcga tattcgcgcc 3240
gacttcgccc ttggcggtat taccgcgacg atggttgacc tgcacgaaaa aggtctgatc 3300
cgcaaactgc tggatgtgca gagctttgac agccatgctg cgcaatcgct ggcccgtaac 3360
cccaatcaca tcgaaatcag cgccaaccag tacgctaact ggggttcgaa aggcgcatcg 3420
gttgatcgtc tcgacgtggt ggtactgagc gcgctggaaa ttgacaccca gttcaacgtt 3480
aacgtgctga ccggctctga cggcgtactg cgtggtgctt ccggtggtca ctgcgatacc 3540
gcgattgcct ctgcgctttc catcatcgtc gcgccgctgg tacgcggtcg tattccgact 3600
ctggtggata acgtactgac ctgcatcacc ccaggctcca gtgtcgatat tctggtcaca 3660
gaccacggta tcgcagttaa cccggcacgt ccggaactgg cagaacgtct gcaggaagcg 3720
ggcattaaag tggtttccat tgagtggctg cgcgaacgtg cgcgtctgct gaccggtgaa 3780
ccacagccga ttgaattcac agaccgcgtc gttgccgttg tgcgttaccg cgatggctcg 3840
gtgatcgatg ttgtgcatca ggtgaaggaa taagccatgc acctgcttcc tgaactcgcc 3900
agccaccatg cggtatcaat tcccgagctg ctcgtcagcc gggatgaaag gcaagcacgg 3960
caacacgtct ggctcaagcg ccatcctgtt ccactggtct cctttaccgt ggttgcgcct 4020
gggccgatta aagacagcga ggtcacacgc cgaattttta atcatggcgt gacagccttg 4080
cgtgccttag ccgcaaaaca gggctggcaa attcaggagc aggctgcact ggtttccgcc 4140
```

agcgggccgg agggcatgtt gagcattgcc gccccggctc gcgacctcaa gctcgccacc 4200
attgagcttg aacatagtca tcctctcggg cggttatggg atatcgatgt cctgacgccc 4260
gaaggcgaaa ttctctcccg ccgcgactat tcactgccgc ctcgccgctg cctgttgtgc 4320
gaacaaagcg cagccgtctg cgcgcgtgga aaaacccatc aactgaccga tttactcaac 4380
cgcatggagg cactgctgaa cgatgtcgat gcctgcaacg tcaactaaaa ccacaaagct 4440
tgcgacgtca ttaatcgatg agtacgccct gctgggctgg cgcgccatgc tgactgaagt 4500
caatctgtca ccgaaaccag gcctcgtgga tcgcattaac tgcggtgcgc acaaagatat 4560
ggcgctggaa gatttccacc gcagcgcgct ggcgattcag ggctggctac cccgtttcat 4620
tgaatttggt gcctgtagtg cggaaatggc accagaagcg gtactccacg gattacgccc 4680
aattggtatg gcttgcgaag gtgatatgtt ccgcgccact gcgggcgtaa acacgcataa 4740
aggcagcatt ttttctttag ggctgctatg tgcggcaatt ggccgtttgc ttcaactcaa 4800
ccaaccggta acgccaacaa ccgtttgttc tacggcggca agtttctgcc gtggcctgac 4860
cgatcgcgaa ctgcgtacca ataattcaca actgacggca ggtcaacggt tgtaccaaca 4920
gcttggcctt accggcgcac gcggtgaagc cgaagcgggt tatccactgg tgatcaatca 4980
cgccttgccg cattacctca ctctgctgga tcaggggtta gatcctgaac tggcattgct 5040
cgataccttg ctcctactga tggcgatcaa cggcgatacc aacgttgcat cgcgcggtgg 5100
cgaggggggc ctgcgctggc tacagcgcga ggcgcaaaca ttattgcaaa aaggggggcat 5160
tcgaaccccc gccgatctcg attatctccg gcagttcgac agggagtgta tcgaacgaaa 5220
tctcagtcca ggcggcagtg ctgacctact gatccttacc tggtttttag cacagattta 5280
attatttaag cacttgataa atttggaaat attaattttc ggagaacccg tatgtcttta 5340
gcaaaagata atatatggaa actattggcc ccactggtgg tgatgggtgt catgtttctt 5400
atccctgtcc ccgacggtat gccgccgcag gcatggcatt acttcgctgt gtttgtggca 5460
atgattgtcg gcatgatcct cgag 5484

<210> 4
<211> 33
<212> DNA
<213> E. coli

<400> 4
aaatttcata tgcacctgct tcctgaactc gcc 33

<210> 5
<211> 36
<212> DNA
<213> E. coli

<400> 5
gggcccctcg agttagttga cgttgcaggc atcgac 36

<210> 6
<211> 552
<212> DNA
<213> E. coli

<400> 6
atgcacctgc ttcctgaact cgccagccac catgcggtat caattcccga gctgctcgtc 60
agccgggatg aaaggcaagc acggcaacac gtctggctca agcgccatcc tgttccactg 120
gtctcctttа ccgtggttgc gcctgggccg attaaagaca gcgaggtcac acgccgaatt 180
tttaatcatg gcgtgacagc cttgcgtgcc ttagccgcaa aacagggctg gcaaattcag 240
gagcaggctg cactggtttc cgccagcggg ccggagggca tgttgagcat tgccgccccg 300
gctcgcgacc tcaagctcgc caccattgag cttgaacata gtcatcctct cgggcggtta 360
tgggatatcg atgtcctgac gcccgaaggc gaaattctct cccgccgcga ctattcactg 420

ccgcctcgcc gctgcctgtt gtgcgaacaa agcgcagccg tctgcgcgcg tggaaaaacc 480
catcaactga ccgatttact caaccgcatg gaggcactgc tgaacgatgt cgatgcctgc 540
aacgtcaact aa 552

<210> 7
<211> 5593
<212> DNA
<213> Klebsiella pneumoniae

<400> 7
ttaattaaca acataaaaac cataaagcca attaagccac gagaaaaact gtgacttaaa 60
tacaagaatc catagccgaa cgctggcgaa atacagttcg ttttgaaatg acgaagcgct 120
aaaaaatgac actgatatta aaacgcgttc agctattaaa agataaaccg cggcgagagg 180
cgatcgatcg gtttctccgc cagcatcaac tgtcgttaga ggccgactgc gaaatggcga 240
ttatcgccga gtatcagcag cggctggtcg gctgcggtgc tatcgccggc aatgtgctga 300
aatgcatcgc catcgatccc tcgctgcagg gggaggggct gagccttaaa ttactgaccg 360
agctcctgac gctggcctat gagctggggc gcagcgaact gtttttgttc actaaacctt 420
gcaatgccgc gttattttcc ggcgccggct tctggccgat agcccaggcg ggcgaccgcg 480
ccgtgctaat ggaaaatagc cgcgaacggc tgactcgtta ctgtcgacag ctggcgatgt 540
accgtcagcc gggaagaaaa atcggcgcta tcgtgatgaa tgctaatcca ttcaccctcg 600
gccaccgctg gttggtagaa caggcggcca gccagtgcga ctggctgcat ctgtttgtgg 660
tcaaagaaga tgcgtcctgc ttttcctatc acgatcgctt caagctcatt gaacagggga 720
ttaccggcat cgataaggtg acgctgcatc ccggttcggc gtatctgatc tcgcgggcga 780
cgttccccgg ctatttcctg aaagagcagg gggtggttga tgactgccac agccagattg 840
acctgcagct cttccgcgag cgcctggccc cggcgctgca gattacccat cgctttgtcg 900
gcaccgagcc gctgtgtccc ctgacccgta attacaacca gcgcatgaag tcactactgg 960
aagcgccagg cgacgcgccg cccattgaag tagttgagct tgcgcgaatc gaaaaaaatg 1020
gtggacccgt gtcggcctcc cgagtgcgcg aactctatcg acagcgcaac tggcaggcgg 1080
tcgcggcgct ggtaccgccg ggaaccctct cttttctgat gcaactggcg gaaagcgaac 1140
atcaaaccgc ctgatttata cgccctaact aaggattttc ccctatggaa atgaagattg 1200
acgccctggc cggcacgctg gagtccagcg atgtgatggt caggattgga cccgcggcgc 1260
agccgggcat tcagctggaa atcgacagca ttgtgaaaca acagtttggc gctgcgattg 1320
agcaggtagt gagagaaacg ctggctcagc ttggcgtgaa acaggccaac gtggtggtcg 1380
atgataaagg cgcgctggaa tgtgttttgc gagctcgcgt acaggccgcg gcgctgcgcg 1440
cggcgcaaca gacccaatta caatggagcc agctatgaaa ccacgtcgca gtatgttgtt 1500
catccctggc gccaatgccg ccatgttaag cacgtcattc gtctacggcg ctgatgctgt 1560
gatgttcgac ctggaagatg ccgtttcgct gcgcgagaaa gataccgctc gtctgctggt 1620
gtatcaggcg ctgcagcatc cactgtatca ggatatcgaa accgtggtgc gtattaaccc 1680
gctaaatacc ccgtttggtc tggccgatct ggaagccgtg gttcgtgcgg gcgtggatat 1740
ggtgcgtctg ccgaaaaccg acagcaaaga agatatccat gagctggaag cgcatgttga 1800
gcggattgaa cgcgagtgcg gccgggaagt gggcagcacc aagttaatgg cggcgatcga 1860
gtcggcgctg ggcgtggtga acgcggtgga aatcgcccgc gccagcccgc gtctggcggc 1920
gatcgcgctg gcggccttcg attacgtgat ggatatgggc acctcccgcg gcgacggtac 1980
tgaactgttc tacgcccgct gcgctgtact gcatgccgcc cgcgttgccg gcatcgccgc 2040
ctatgacgtg gtgtggtcgg atatcaataa tgaagagggc ttcctggcgg aagcgaatct 2100
ggccaaaaac ctcggcttta acggcaaatc gttggttaac ccacgacaaa ttgaactcct 2160
gcatcaggtc tatgccccga cgcgcaaaga ggtcgatcac gcgctggaag tgattgccgc 2220
ggcggaagaa gccgaaacgc gaggtctggg tgtggtatcg ctgaacggca agatgatcga 2280
tggaccgatt atcgaccatg ctcgcaaagt ggtggcgctc tcggcttccg gtattcgtga 2340
ttaaggggaa taagatgaaa gagacagtag caatgcttaa tcagcagtac gtgatgccga 2400
atggactgac accttatgcc ggcgtaacgg cgaaaagtcc ctggctggcg agtgagagcg 2460
aaaagcgcca gcgcaaaatc tgcgattcgc tggaaacggc aatccgtcgc tccggcctgc 2520
aaaacggcat gaccatctcg tttcaccacg cgtttcgcgg cggtgacaaa gtcgtcaata 2580

```
tggtagtggc gaagctggcg gaaatgggtt ttcgcgatct caccctggcg tccagttcgc 2640
tgatcgacgc ccactggccg ctgatcgagc atattaaaaa tggcgtgatc cgccagatct 2700
acacctccgg cctgcgcggc aagttgggcg aggagatctc cgccggttta atggaaaacc 2760
cggtgcagat ccactcccac ggcggtcgcg tacagctgat tcaaagcggc gagctgtcga 2820
ttgatgtcgc gtttctcggc gttccttgct gcgatgagtt tggcaacgcc aacggcttta 2880
gcggtaaatc acgctgcggt tctctgggct acgcgcgcgt cgatgccgag cacgctaaat 2940
gcgtggtgct gctcaccgaa gagtgggtgg attatcctaa ctatccggcc agtattgccc 3000
aggatcaggt ggatctgata gtccaggtag atgaagtcgg cgatccgcaa aaaattaccg 3060
cgggtgccat ccgtctgacc agcaacccgc gcgagctgct gatcgcccgc caggcggcga 3120
aagtcgttga gcactccggt tactttaaag agggtttctc gctgcagacc ggtaccggcg 3180
gcgcctcgct ggcagtaact cgcttccttg aagataaaat gcgccgtaac ggcattaccg 3240
ccagcttcgg cctcggcggt atcaccggga cgatggtcga tttgcacgaa aaagggttga 3300
tcaaaacgct gctcgatacc cagtccttcg atggtgacgc ggcgcgttcg ctggcgcaga 3360
acccgaacca tgtcgagatc tccaccaatc agtatgccag cccgggctcc aaaggcgcct 3420
cctgcgagcg cttaaacgtg gtgatgctca gcgcgctgga aattgatatc gactttaacg 3480
ttaacgtgat gaccggttct aacggtgtgc tgcgcggggc gtccggtggc catagcgata 3540
ccgccgccgg tgcggatttg accattatta ccgcgccgtt agttcgcggc cgtattccct 3600
gcgtcgtgga aaaggtgctg acccgcgtca cgccgggggc cagcgtggat gtgctggtca 3660
ctgaccacgg cattgcggtc aacccggcac gtcaggacct gatcgacaat ttgcgcagcg 3720
caggcattcc gctgatgacc attgaggaac tgcagcagcg tgctgagctg ttgactggca 3780
agccgcagcc gatcgaattc accgatcggg tggtggcggt ggtgcgctat cgcgacggtt 3840
cggtcatcga tgtgattcgt caggtgaaaa acagcgacta aacgcagagg ggaaaggcca 3900
tgagcgacgt gttaattaat cctgcgcgtg tgcggcgcgt gaagccactg agtgccgaag 3960
aggtggtcag cgcggtagag cgcgcgctgt tgaccgaagt tcgcctgacc ccaaagcccg 4020
ggttggtgga tattcgtaac gctggcgcgc actgggatat ggatctggcc tcgtttgagg 4080
ccagcaccgc ggtggtggct ccgtggatgg agaaattttt catcatgggc cacgatactg 4140
cggcggtcgc gccggagcag gtattgatga tgctgcgccc ggtagggatg gcctgtgaga 4200
acgatatgct ggaggccacc ggcggggtga atacccatcg cggggcgatc ttcgcttttg 4260
gcctgctcag cgcggcggcg ggcaggctgg tgtcgaaagg tgagccgata gagcagcacc 4320
ggctttgcga ccaggtggcg cgcttctgtc gcggcatggt tatgcaggag ttgtcttctg 4380
ctggcgggga acggctcagt aaaggcgagg ctcattttct acgctatggt ctctccgggg 4440
cccgcggcga ggcggagagc ggtttcctga cggtgcgtac ccaggccatg ccagtcttta 4500
cccgcatgat ggaagagacc ggcgacagta atctggcgct actgcaaacc ctgctgcatc 4560
tgatggcgtg gaatgatgac accaacctgg tctcgcgcgg cgggcttgcc gggctgaact 4620
ttgtccagca ggaggcgcag cgactgctgt ggcagggcgg cgtgctggcg gacggcgggc 4680
tggaggcgct gcgacagttt gacgatgagc tgattgcccg ccatctcagc cctggcggca 4740
gcgccgatct gttggcggtg acctggtttt tatccgcgtt tcccgccggc gcgcttttcc 4800
cgctgtaacc cactgcaata ccgccttcgc ccgcactgta cgggcgaggg cgccatcatt 4860
agccttcccg gttgtcatcc ggtaaacacg gaatcgcggc acaatcgtat agtttttact 4920
gatatcgtcc gccgtttgtc ataaatttct aattatcggc gtttttgagt agcggcccgc 4980
tgacgggctg gttactctga aaacaattta cgtaatgtta acaaaagaga atagctatgc 5040
atgatgcaca aatccgcgtg gccatcgccg gcgcgggcgg ccggatggga cgccagttaa 5100
ttcaggctgc attgcagatg gaaggcgtgg cgctgggcgc ggcgctggag cgcgaagggt 5160
caagcctggt gggcagcgac gccggcgagc tggcgggcgc cggcaaagcg ggcgtcgcgg 5220
tgcagagcag cctggcggcg gtaaaagatg atttcgacgt gttgatcgat tttacccgcc 5280
cggaaggcac gctgaaccat ctggcgtttt gccgcgagca cggcaaaggg atggtcatcg 5340
gcaccaccgg ttttgacgac gctggcaaac aggcgattcg cgatgccgcg caggacattg 5400
ccattgtctt cgccgctaac tttagcgttg gcgtcaatgt cctgttgaag ctgctggaga 5460
aggcggcgaa ggtgatgggc gactataccg acatcgaaat tatcgaagcg caccaccggc 5520
ataaagtgga tgcgccgtca ggcaccgcgc tggcgatggg cgaagcgatc gccggggcat 5580
tgaacaaaga tct                                                    5593
```

Claims

1. Process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organism and isolating the protein in an active form, wherein the plasmid contains the information from a gene cluster composed of at least six genes and an inducible promoter.

2. Process as claimed in claim 1, wherein the genes code for certain subunits of the protein having citrate lyase activity and/or for components that contribute to the biosynthesis of the complete enzyme.

3. Process as claimed in one of the claims 1 or 2, wherein the plasmid contains the genes citC, citD, citE, citF, citG and a DNA fragment obtainable from E. coli that is located between citF and citG on the E. coli citrate lyase gene cluster.

4. Process as claimed in claim 3, wherein the DNA fragment codes for a 20 kDa protein.

5. Process as claimed in claim 3 or 4, wherein the DNA fragment codes for a protein containing the motif G(A)-R-L-X-D-L(I)-D-V.

6. Process as claimed in one of the claims 1 to 5, wherein at least one gene is obtainable from E. coli, Haemophilus influenzae, Klebsiella pneumoniae or Leuconostoc mesenteroides.

7. Process as claimed in one of the claims 1 to 6, wherein at least four genes are derived from the microorganism that is specific for the isolated protein with citrate lyase activity.

8. Process as claimed in claim 7, wherein it is Klebsiella pneumoniae.

9. Process as claimed in one of the claims 1 to 8, wherein the host organism is a eukaryotic or prokaryotic microorganism.

10. Process as claimed in claim 9, wherein it is E. coli.

11. Process as claimed in one of the claims 1 to 10, wherein the expression occurs under aerobic conditions.

12. Recombinant soluble protein with citrate lyase activity and a molecular weight of about 14,000 to 15,000 Dalton obtainable by a process as claimed in one of the claims 1 to 11.

13. Test kit for the determination of citric acid which comprises essentially the following components

(a) a protein with citrate lyase activity obtainable according to one of the claims 1 to 11,
(b) at least one protein with hydrogen-transferring activity
(c) nicotinamide adenine dinucleotide or a corresponding derivative in a reduced form and
(d) optionally suitable stabilizers, activators and/or substances to avoid or reduce interferences, and buffer solutions.

14. Test kit as claimed in claim 13, wherein L-malate dehydrogenase and optionally L-lactate dehydrogenase are used as the hydrogen-transferring enzymes.

15. Use of the enzyme obtainable according to claims 1 to 11 to determine citric acid.

Abstract

Process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organism and isolating the protein in an active form, wherein the plasmid contains the information from a gene cluster composed of at least six genes and an inducible promoter. Furthermore the invention concerns the use of the recombinant enzyme and a corresponding test kit for the determination of citric acid.

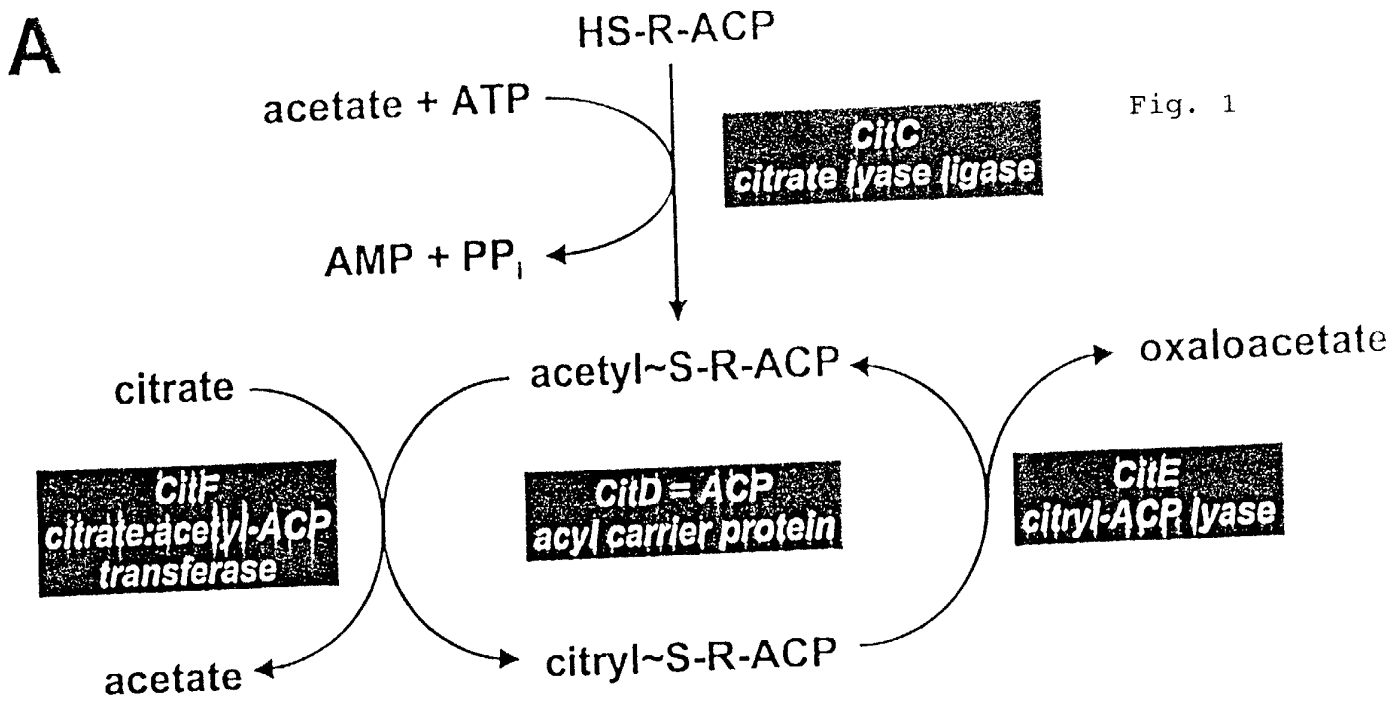
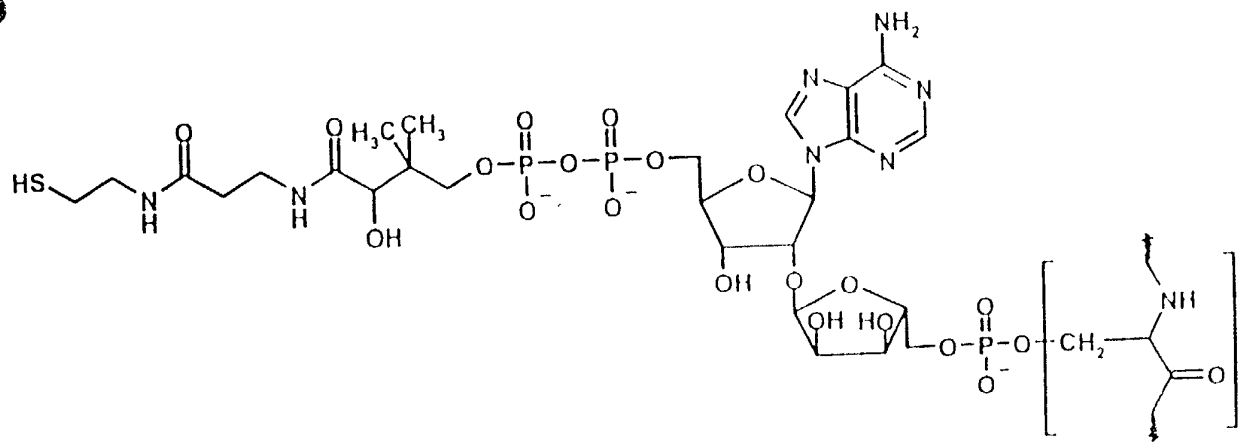


Fig. 1

B



serine-14 residue of the acyl carrier protein

Fig. 2

1 kb

K.p.

Ligase Citrate lyase

E.c.

Ligase Citrate lyase

H.i.

Ligase Citrate lyase

L.m.

Ligase Citrate lyase

Docket No.
BMID9975US

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
99119404.4	Europe	30 September 1999	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Kenneth J. Waite, Reg. No. 45,189
D. Michael Young, Reg. No. 33,819
Brent A. Harris, Reg. No. 39,215
Marilyn L. Amick, Reg. No. 30,444
Jill Lynn Woodburn, Reg. No. 39,874

Send Correspondence to: **Kenneth J. Waite**
Roche Diagnostics Corporation
9115 Hague Road, Bldg. D, P.O. Box 50457
Indianapolis, IN 46250-0457

Direct Telephone Calls to: *(name and telephone number)*
Kenneth J. Waite, Telephone No. (317) 576-3104

Full name of sole or first inventor **BOTT, Michael**
Sole or first inventor's signature Date
Residence **Heinsberger Strasse 2, D-52428 Juelich, Germany**
Citizenship **German**
Post Office Address **(same as residence)**

Full name of second inventor, if any **DIMROTH, Peter**
Second inventor's signature Date
Residence **Irisweg 3, CH-8700 Kuesnacht, Switzerland**
Citizenship **German**
Post Office Address **(same as residence)**

Full name of third inventor, if any **SCHNEIDER, Karin**
Third inventor's signature Date
Residence **Altwiesenstrasse 64, CH-8051 Zuerich, Switzerland**
Citizenship **Swiss**
Post Office Address **(same as residence)**

Full name of fourth inventor, if any
Fourth inventor's signature Date
Residence
Citizenship
Post Office Address

Full name of fifth inventor, if any
Fifth inventor's signature Date
Residence
Citizenship
Post Office Address

Full name of sixth inventor, if any
Sixth inventor's signature Date
Residence
Citizenship
Post Office Address